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Development of a Porous Silicon Based Biosensor

Keiki-Pua S. Dancil, Douglas P. Greiner, and Michael J. Sailor*
Department of Chemistry and Biochemistry, The University of California San Diego, La Jolla, CA 92093-0358

ABSTRACT

In this paper we demonstrate that porous silicon (PS) can be used as an immobilization matrix and a transducer for biosensor applications. Thin layers of PS were fabricated showing fine structure in their reflection spectra, characteristic of longitudinal optical cavity modes, or Fabry-Perot interference fringes. The PS surface was modified by covalently bonding streptavidin to a heterobifunctional linker immobilized to the surface using common silane chemistry. The mode spacing and wavelength in the interference spectrum was modified, by displacing buffer and introducing proteins into the PS layer. Protein-protein interactions between immobilized Streptavidin and biotinylated Protein A followed by Protein A and IgG were detected. The surface was regenerated during the course of the experiment showing reversibility of the sensor at the third layer.

INTRODUCTION

Inexpensive biosensors designed to provide rapid multi-analyte detection are highly sought after for use in drug design and disease diagnosis, as well as detection of chemical and biological warfare agents. The basic design of most biosensors involves the incorporation of two components: a specific analyte recognition element (typically an immobilized biomolecule, i.e. oligonucleotide or protein) and a transducer which transforms a molecular recognition event into a quantifiable signal. Recently, our laboratories reported the discovery of a system which utilizes PS as an immobilization matrix for biomolecules and as an optical interferometric transducer of molecular binding events [1]. In addition to its unique optical and chemical properties, PS was chosen as the matrix material due to its high surface area. In comparison to flat surfaces, PS offers an immense increase in surface area, which allows for a higher amount of immobilized receptor molecules and hence increased sensitivity.

The sensor is based upon changes in the refractive index of the thin PS film. When incident white light is reflected from a PS sample, Fabry-Perot fringes in its reflectometric spectrum are observed. The fringe pattern is due to the constructive and destructive interference of light being reflected off the top interface (water/(PS + water)) and the bottom interface ((PS + water)/bulk silicon) of the thin film. The peaks in the interference spectrum ($m\lambda$) are related to the effective optical thickness (EOT) of the film by the following equation: [2]

$$m\lambda = 2nL \qquad (1)$$

where m is the spectral order, λ is the wavelength, n is the refractive index, and L is the thickness of the film. Since the thickness (L) is a constant parameter, changes in the fringe pattern are directly related to changes in the refractive index of the PS matrix (n_{PS}). For example, when a large biological molecule, such as a protein with $n_{protein} \sim 1.42$ is immobilized within the matrix, a corresponding volume of water with $n_{water} = 1.33$ is displaced out of the matrix. The slight increase in the overall n_{PS} causes an increase in the EOT, which is directly observed in the

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interference pattern as a shift to longer wavelengths (red shift). Since this system is solely dependent on refractive index changes in the bulk film, response of the PS biosensor is independent of the analyte to surface distance, unlike sensors employing surface plasmon resonance (SPR).

EXPERIMENTAL

Etching procedure. Porous silicon samples were prepared by anodically etching p^{++}-type silicon (0.6-1.0mΩ-cm resistivity, (100) orientation, B-doped) in an ethanolic HF solution (HF:ethanol 3:1, v/v) at current densities ranging from 400-600 mA/cm^2 in the dark. Etching times varied to obtain constant coulombs of 4.5 C/cm^2. A Pt mesh counter electrode was used to ensure a homogenous electric field. After each etch, the sample was rinsed thoroughly with ethanol and methylene chloride and then dried under a stream of nitrogen.

SEM characterization of PS samples. A Cambridge 360 electron microscope, using an accelerating voltage of 20 keV was used to investigate pore dimensions and geometries. Each sample was sputtered with 10 nm of gold to reduce charging effects.

Derivatization of PS chip. Freshly etched chips were exposed to ozone for 10 minutes using an ozone generator (Fischer) to provide an oxidized surface for further functionalization. SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate (PIERCE) (50 mg) was dissolved in 10 mL of methylene chloride and 4-aminobutyldimethylmethoxysilane (FLUKA) (19 μL) was added while stirring. The reaction was allowed to proceed overnight at room temperature. The product (2-pyridyldithiopropionamido)butyldimethylmonomethoxysilane) was purified via silica gel column using ethyl acetate as the eluent. The purified bifunctional silane was dissolved in 20 mL of toluene and was covalently attached to the oxidized PS surface by overnight reflux. The functionalized PS samples were characterized by FTIR. Total surface coverage was determined by exposure to 10 mM dithiothreitol, DTT, (ALDRICH) and detection of pyridine-2-thione by UV/VIS spectroscopy (343 nm, $\varepsilon = 8.08 \times 103\ M^{-1}cm^{-1}$) [3].

Streptavidin immobilization. 2 mg of streptavidin (PIERCE) was dissolved in 0.5 mL of 0.5 M sodium phosphate, pH 8.0. After cooling the protein solution on ice, 22 μL of 100 mM 2-iminothiolane (2-IT) (dissolved in water) was added and the reaction was allowed to proceed for 30 minutes at 0° C. To remove excess 2-IT, the modified protein was purified by centrifuge spin column (Sephadex G-50 equilibrated in phosphate buffer saline (PBS) pH 7.4 [4]). The purified protein solution was combined with an equivalent volume of 20% (v/v) ethanol in PBS. The diluted streptavidin solution in ethanol was introduced to a PS chip, mounted in a flowcell, pre-equilibrated with PBS containing 10% (v/v) ethanol at a flow rate of 0.5 mL/min. Interferometric spectra were obtained and analyzed as described by Janshoff *et al.* [5].

Protein binding. Biotinylated Protein A (PIERCE) was dissolved in PBS pH 7.4 to a concentration of 2.5 mg/mL. Human IgG (CALBIOCHEM) was solubilized in PBS pH 7.4 at a concentration of 1.0 mg/mL. Each protein solution was introduced to the modified PS at a flowrate of 0.5 mL/min.

DISCUSSION

For biosensor applications it is essential that the pore dimensions be large enough to allow for diffusion of proteins in and out of the pores, but small enough to preserve reflective optical properties. By adjusting the current density, HF concentration, and dopant density of the

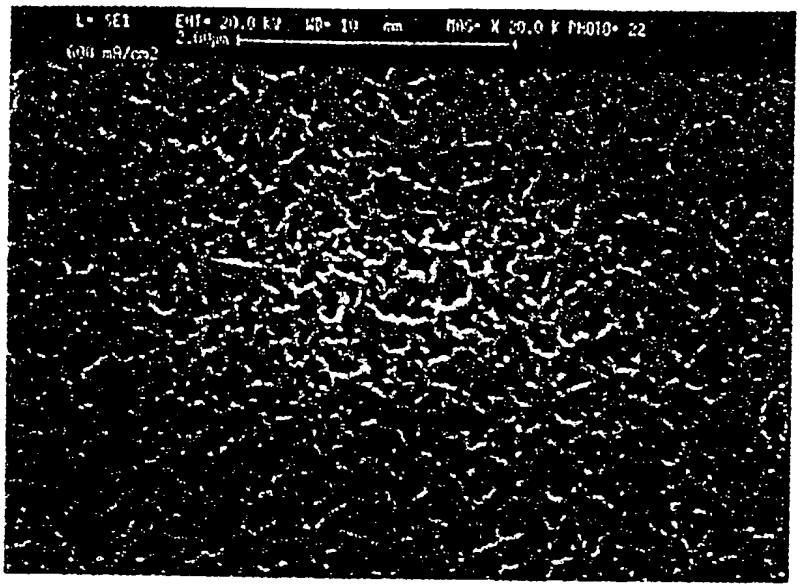

Figure 1. SEM top view image of a PS chip etched at 600 mA/cm^2.

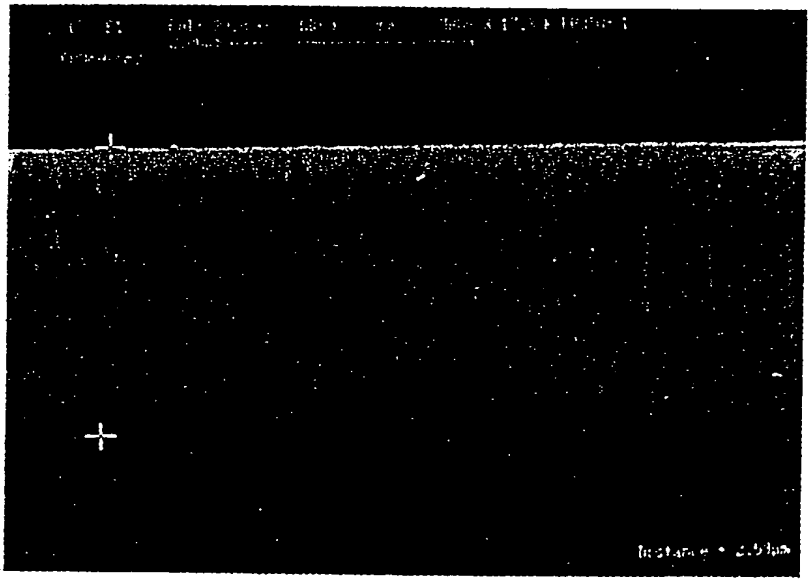

Figure 2. SEM cross sectional image of a PS chip etched at 600 mA/cm^2.

silicon, PS samples were fabricated having applicable dimensions. Figure 1 is a top view image of a PS sample etched at 600 mA/cm^2. Pores are visible with radii between 100 – 300 nm. Cross-sectional SEM images were obtained to investigate the pore geometry and depth of the porous layer. Figure 2 is a cross sectional image of the same sample. The pores appear to be 2.5 μm in depth and cylindrical in shape.

Before each binding curve was obtained, a baseline was determined by exposing the surface to PBS (pH = 7.4) containing 10% (*v/v*) ethanol. Ethanol was used to ensure wetting of the hydrophobic porous layer. Streptavidin was covalently attached to the PS matrix via a disulfide bond. Since native streptavidin lacks free cysteines it was necessary to introduce free thiols by reacting free primary amines on streptavidin with Traut's reagent. As shown in part A of Figure 3, an increase of approximately 75 nm in EOT was observed. This clearly indicated covalent immobilization of streptavidin to the surface. A buffer rinse of PBS, pH 7.4, containing 10% (*v/v*) ethanol ensured removal of non-covalently attached protein. After immobilization ethanol was removed by passing PBS over the sample. The removal of ethanol, at point B, is evident by a decrease in EOT due to the reduced refractive index of the PBS buffer. At point C, biotinylated Protein A was introduced. The EOT increased by 25 nm, indicative of an interaction between streptavidin and biotinylated Protein A. Control reactions (data not shown) with non-biotinylated Protein A or BSA show no increase in EOT. Human IgG was introduced at point D, showing an observed EOT change of approximately 11 nm. Acetic acid (1.0 M) was used at points E to selectively remove the bound IgG. The increase in EOT was due to higher refractive index of acetic acid buffer verses that of PBS. However, a PBS rinse at point F restored the curve to a baseline expected for a biotinylated Protein A surface. This decrease in EOT is characteristic of the released IgG. The procedure of adding IgG (point G), acetic acid (point H), and PBS rinse (point I) gave similar results. At point J, 10 % (*v/v*) ethanol in PBS was added followed by the addition of 10 mM DTT in 10% (*v/v*) ethanol in PBS (point K). DTT was added to reduce the disulfide bonds, removing all proteins from the surface. Figure 4 is an expanded view of Figure 3 showing the reversible interactions of the Protein A and Human IgG complex on the sensor surface.

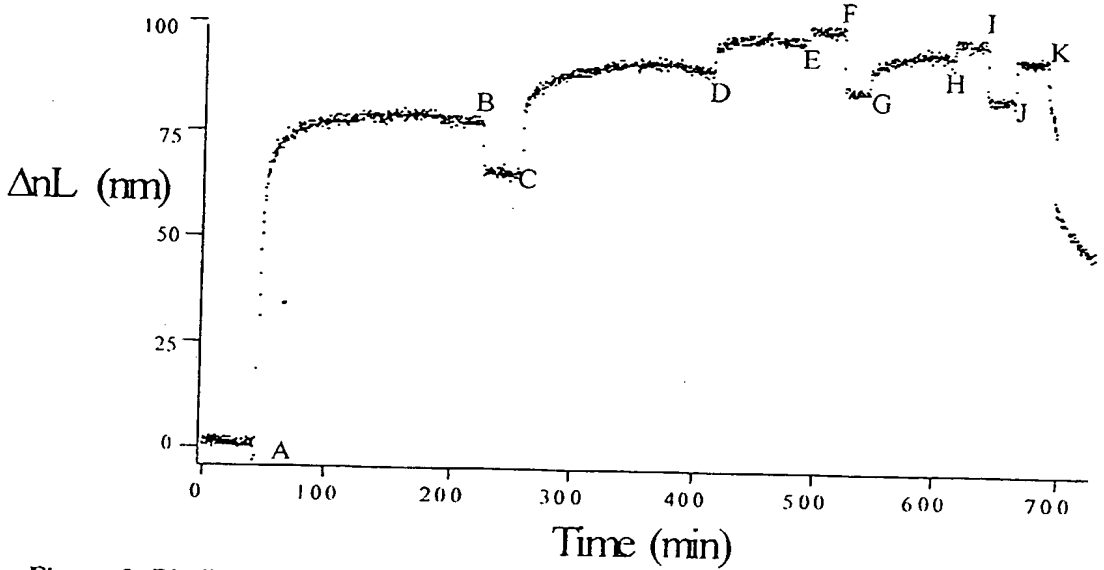

Figure 3: Binding curve showing changes in EOT over the course of an experiment. Between each step a buffer rinse step was conducted using PBS pH 7.4

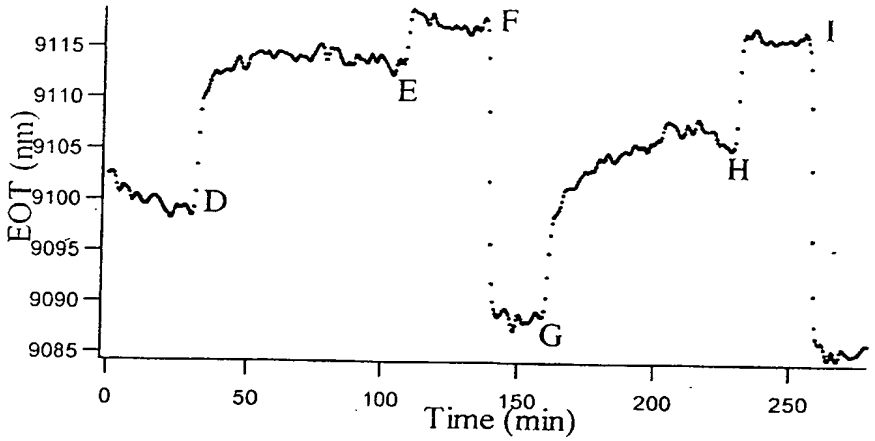

Figure 4: An expanded version of Figure 3 showing the reversibility of the sensor surface.

The sensor is based on changes in refractive index. Because most proteins have a refractive index of approximately 1.45, the PS sensor can be used to monitor changes in molecular weight. In the first layer an increase of 75 nm was detected by immobilization of streptavidin (MW = 60,000 g/mol). The next layer only showed an EOT change of 25 nm for biotinylated Protein A (MW = 42,000 g/mol). By introducing Human IgG (MW = 155,000 g/mol) to the surface, an EOT increase of 11 nm was detected. Each layer of Human IgG introduced gave similar changes in EOT. The mass of streptavidin and biotinylated Protein A are similar and should show equal changes in EOT. But, biotinylated Protein A gave a signal of one third that of streptavidin. We attribute the increased signal of streptavidin to wetting of the surface. After the first protein layer is bonded to the surface, it becomes more hydrophilic in character, allowing further layers of protein to interact. The molecular weight of Human IgG is approximately three times that of Protein A. Therefore, Human IgG should have shown an EOT increase three times that of Protein A. The signal from Human IgG was only half the signal of Protein A. We attribute the lower than expected shifts in signal to size exclusion effects; the large IgG protein is not able to access all of the available Protein A sites in the confined pores of the PS film.

CONCLUSIONS

We have demonstrated the use of PS as a transducing and immobilization matrix for biosensing using Protein A as a reversible binding substrate. Furthermore, we have detected reversible binding at the third protein layer of the sensor surface.

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